PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

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(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing: 11 September 1998 (11.09.98)	
International application No.: PCT/EP98/01293	Applicant's or agent's file reference: O/97263 WO
International filing date: 03 March 1998 (03.03.98)	Priority date: 05 March 1997 (05.03.97)
Applicant: MEULEMAN, Dirk, Gerrit et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

14 August 1998 (14.08.98)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
0/97263 WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 98/ 01293	03/03/1998	05/03/1997
Applicant		
AKZO NOBEL N.V. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application.
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:
   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 98/01293

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 5, 6
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 5, 6 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/01293

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/565

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAENGGI ET AL.: "postmenopausal hormone replacement therapy with tibolone decreases serum lipoprotein(a)" EUR. J. CLIN. CHEM. CLIN. BIOCHEM., vol. 31, 1993, pages 645-650, XP002036506 see the whole document ---	1-6
X	RYMER ET AL: "effects of tibolone on serum concentrations of lipoprotein(a) in postmenopausal women" ACTA ENDOCRINOLOGICA, vol. 128, 1993, pages 259-262, XP002036507 see the whole document --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 1998	12/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Trifilieff-Riolo, S

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KLOOSTERBOER ET AL: "long-term effect of Org OD 14 on lipid metabolism in post-menopausal women" MATURITAS, vol. 12, 1990, pages 37-42, XP002036508 see the whole document ---	1-6
X	VOLPE ET AL: "benefits and risks of different hormonal replacement therapies in post-menopausal women" MATURITAS, vol. 8, no. 4, 1986, pages 327-334, XP002036515 see the whole document ---	1-6
A	RIGGS: "tibolone as an alternative to estrogen for the prevention of postmenopausal osteoporosis" J. OF CLINICAL ENDOCRIN. AND METABOLISM, vol. 81, no. 7, 1996, pages 2417-2418, XP002036516 cited in the application see the whole document -----	1-6

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(22) International Filing Date: 3 March 1998 (03.03.98)

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(34) Countries for which the regional or international application was filed: NL et al.

(71) Applicant *(for all designated States except US)*: AKZO NOBEL N.V. [NL/NL]; Velperweg 76, NL–6824 BM Arnhem (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: MEULEMAN, Dirk, Gerrit [NL/NL]; Beethovengaarde 69, NL–5344 HC Oss (NL). ZANDBERG, Pieter [NL/NL]; Uitstroom 1, NL–5345 RX Oss (NL).

(74) Agent: HOGENBIRK, M.; P.O. Box 20, NL–5340 BH Oss (NL).

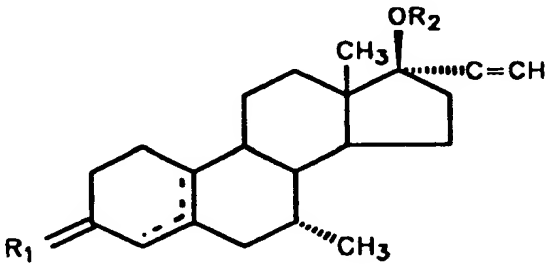
(81) Designated States: AM, AU, BB, BG, BR, CA, CN, CZ, EE, GE, HU, ID, IS, JP, KG, KP, KR, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TR, TT, UA, US, UZ, VN, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF A 7α–METHYL–17α–ETHYNYL–ESTRANE DERIVATIVE FOR THE TREATMENT OF ATHEROSCLEROSIS

(57) Abstract

The invention relates to the use of a 7α–methyl–17α–ethynyl–estrane derivative having general formula (I) wherein R_1 = H(OR_3) or O; R_2 = H or (C_{1-18})Acyl: R_3 = H or (C_{1-18})Acyl; and the dotted line represents a double bond in the 4,5– or the 5,10–position for the manufacture of a medicament for the prophylaxis or the treatment of atherosclerosis.



(I)

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/01293

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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Trifilieff-Riolo, S

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INTERNATIONAL SEARCH REPORT

..ternational application No.
PCT/EP 98/01293

Box I Observations wh re certain claims w re found unsearchabl (Continuation of item 1 f first sh et)

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1. [X] Claims Nos.: 5, 6
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 5, 6 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

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4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees wer accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

USE OF A 7α-METHYL-17α-ETHYNYL-ESTRANE DERIVATIVE FOR THE TREATMENT OF ATHEROSCLEROSIS

The present invention relates to the use of a 7α-methyl-17α-ethynyl-estrane derivative for the manufacture of a medicament for the prophylaxis and the treatment of atherosclerosis.

The development of atherosclerosis starts with the accumulation of cholesterol in lipoproteins in the vessel wall and the subsequent development of fatty streaks (probably the earliest macroscopically recognizable lesions), which appear in the intima of the arterial vessel wall as focal collections of lipid-filled macrophages ("foam cells"). This process can progress in the formation of advanced lesions; foam cell necrosis and endothelial damage can occur leading to smooth muscle cell migration and proliferation, and to the formation of extracellular matrix. Thus atherosclerosis is the result of th interaction of a number of cell types in the vessel wall, in which increased plasma cholesterol can be the driving force (Davies, M.J., and Woolf, N.; "Atherosclerosis: what is it and why does it occur"; Brit. Heart J., 1993, 69 (suppl.), S3-S11).

Coronary heart disease (CHD) is a consequence of atherosclerotic processes in the artery vessel wall. It is well known that the incidence of CHD in women in the reproductive stage of life is much lower than in men of similar age but that the risks sharply increase following the menopause.

The menopause has been associated with a large number of vasomotor, psychological and gynecological symptoms, part of which are characteristic for the perimenopausal period (climacteric). The menopause has been shown to be a risk factor for chronic diseases like osteopor sis and ath rosc l rosis. Th sharply decr asing concentrations of strogens, specially of stradiol (estra-1,3,5 (10)-triene-3,17-diol) and estron (3-hydroxy-estra-1,3,5 (10)-

triene-17-one), in post-menopausal women have been suggested to be related to these symptoms. Estrogen replacement therapy, through which the physiologic deficit of mainly estrogen is to be corrected, is gaining increasing acceptance as a means of alleviating climacteric symptoms in peri- and post-menopausal women and preventing osteoporosis. In addition, exogenous estrogen is reported to have a plasma cholesterol- and a LDL (low density lipoproteins)-cholesterol lowering effect and/or a plasma HDL (high density lipoproteins)-cholesterol increasing effect. These estrogen effects may be suggestive of a protective overall effect on the formation of atherosclerotic lesions.

However, clinically unopposed estrogen replacement therapy in post-menopausal women can increase the risk of endometrial hyperplasia and endometrial cancer. Therefore most therapies under study to date concern combined treatments with both an estrogen component and a progestagen component, which is added to negate the estrogen mediated risks (hormone replacement therapy). But progestagens can have adverse effects on th plasma lipoprotein concentrations and antagonize the beneficial effects of estrogen on the arterial vessel.

A synthetic steroid, 7α-methyl-17α-ethynyl-17β-hydroxy-estra-5(10)-en-3-one (Org OD-14; tibolone), produced by Organon, The Netherlands, characterized by having a mixed profile of weak estrogenic, progestogenic and androgenic properties, has been shown to be clinically as effective as estradiol valerate or conjugated equine estrogens in reducing climacteric symptoms in peri-menopausal women. Tibolone has further been shown, like the long-term administration of estrogen, to provide substantial protection against the development of osteoporosis in post-menopausal women (Hannover, N. et al., J. Clin. Endocrinology and Metabolism (1996), 81, 2419-2422).

In addition, th eff ct of tibolone on plasma lipoprotein concentrations has been subject of several studies. In arly studies, the overall changes in total

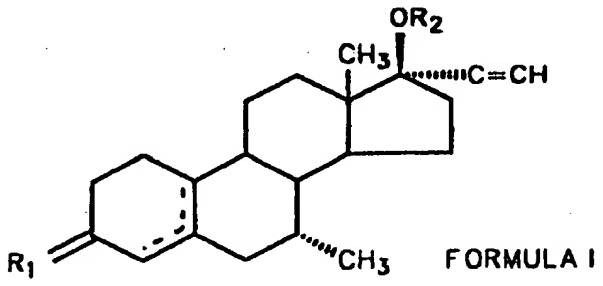
cholesterol, total glycerides or LDL and HDL cholesterol levels as a result of tibolone treatment after a 6-month period were reported to be not significant, implying that no negative side effects with respect to the early development of CHD were to be expected. Therefore, the compound was considered a useful alternative to conventional hormonal replacement therapy in post-menopausal women. (Maturitas, vol.8, no.4, 1986, pages 327-334). Studies assessing the long-term effects of tibolone treatment on lipid metabolism confirmed these findings. (see e.g. Maturitas, vol.12, no.1, 1990, pages 37-42). Other studies emphasize the negative effect of tibolone on HDL levels (a decrease, which is associated with an increased CHD risk), but describe a benificial decreasing effect found on lipoprotein(a) (Lp(a)) levels, which according to the authors may help to restore the balance of (cardiovascular) risks associated with tibolone therapy. (Acta Endocrinologica, vol. 128, 1993, pages 259-262; Eur. J. Clin. Chem. Clin. Biochem., Vol. 31, 1993, pages 645-650).

[Lp(a) is a cholesterol-rich lipoprotein which resembles LDL but is present only in trace amounts in most individuals. Those with elevated (>300 mg/l) serum Lp(a) concentrations are at high risk of CHD. Lp(a) is an independent risk marker for CHD.]

Nevertheless, the effect of long-term treatment with tibolone on HDL-cholesterol has been interpreted as less favorable with respect to its protective effect against cardiovascular disease, as compared with estrogen replacement therapy (Riggs, B.L., J. Clin. Endocrinology and Metabolism (1996), 81, 2417-2418).

Surprisingly, it has now been found that tibolone, prodrug forms thereof and certain metabolites thereof have strong anti-atherosclerotic properties. These properties are much more pronounced than those of e.g. 17β-stradiol.

The present invention therefore relates to the use of 7α-methyl-17α-ethynyl-estrane derivatives having the general formula I



FORMULA I

wherein

R_1 = H(OR_3) or O; R_2 = H or (C_{1-18})Acyl; R_3 = H or (C_{1-18})Acyl;

and the dotted line represents a double bond in the 4,5- or the 5,10-position, for the manufacture of a medicament for the prophylaxis or the treatment of atherosclerosis.

The compounds of the invention, more specifically the compounds of formula I, wherein R1 is H,OH or O, in particular those wherein R_2 = H and wherein the double bond is at the 5,10-position, and especially the compound wherein R1 is O, R2 is H and wherein the double bond is in the 5,10-position (tibolone; Org OD-14), have a very pronounced atheroprotective effect.

Thus, suitable 7α-methyl-17α-ethynyl-estrane derivatives having general formula I which can be used according to the invention are, for example, 7α-methyl-17α-ethynyl-17β-hydroxy-estra-5(10)-en-3-one (Org OD-14;tibolon), 7α-methyl-17α-ethynyl-estra-5(10)-en-3α,17β-diol, 7α-methyl-17α-ethynyl-estra-5(10)-en-3β,17β-diol, 7α-methyl-17α-ethynyl-17β-hydroxy-estra-4-en-3-one, and esters thereof. Preferred derivatives are tibolone and 7α-methyl-17α-ethynyl-estra-5(10)-en-3α,17β-diol. A particular preferred 7α-methyl-17α-ethynyl-estrane derivativ is tibolone.

Th term acyl means an acyl group d riv d from an organic carboxylic acid having 1-18 carbon atoms, as is also indicated by the affix (C_{1-18}). Examples of such carboxylic acids are formic acid, acetic acid, propionic acid, butyric acid isobutyric acid, trimethylacetic acid, valeric acid, caproic acid, capric acid, undecylenic acid, lauric acid, palmitic acid, oleic acid, phenylacetic acid, phenylpropionic acid, benzoic acid, fumaric acid, maleic acid, succinic acid and citric acid. Preferred acyl groups have 1-6 carbon atoms, and most preferred is the acetyl group.

The 7α-methyl-17α-ethynyl-estrane derivatives according to formula I are known compounds. The compounds can thus be prepared as described, for example, in US Patent 3,340,279 and in US Patent 4,701,450 for 7α-methyl-17α-ethynyl-17β-hydroxy-estra-5(10)-en-3-one (tibolone).

The use of the compounds according to the present invention does not merely lead to an absence of negative cardiovascular side effects in hormone replacement therapy as might be derived from certain prior art sources, but the compounds even have an unexpected, significant and beneficial atheroprotective effect. The 7α-methyl-17α-ethynyl-estrane derivatives according to the invention, and pharmaceutical preparations based thereon, have a beneficial effect on the cholesterol accumulation in the vessel wall, the fatty streak formation and the advanced lesion formation.

These direct effects on the vessel wall were observed in a general accepted, relevant and validated atherosclerosis model in rabbits. Contrary to expectation, in view of the weak estrogenic activity of the compounds of the invention, the remarkable and unpredicted strong atheroprotective effect of the compounds of the invention has been demonstrated down to very low doses and were much stronger in comparison with the atheroprotective eff ct of 17β-estradiol.

Although the compounds of invention -in contrast to estradiol- can in humans lower th plasma concentration of lipoprotein Lp(a), this can not be

th xplanation for th much more pronounced atheroprotectiv ff ct. In contrast to humans in rabbits no Lp(a) is present in th plasma (rabbits do not synthesize Lp(a); see Science, vol. 246, 1989, p.904-910). Therefore the strong atheroprotective effect of the compounds of this invention in (atherosclerotic) rabbits are independent of an effect on Lp(a). Consequently, when in humans a decrease in Lp(a) plays an additional role in atheroprotection, then the unexpected strong atheroprotective effect observed in rabbits might even be stronger in humans.

The 7α-methyl-17α-ethynyl-estrane derivatives of the present invention thus have a strong intrinsic atheroprotective potential and are therefore not only useful drugs in hormone replacement therapy of peri- and post-menopausal women, but they are also suitable for therapeutic use in the treatment of atherosclerosis in mammals, both male and female, of all ages. Additionally, they can also be used prophylactically to prevent atherosclerosis.

The present invention therefore provides a method of inhibiting the process of atherosclerosis comprising administering to a mammal, preferably to a human, an atheroprotective amount of a 7α-methyl-17α-ethynyl-estrane derivative having the general formula I, as previously defined.

The compounds of the present invention may be used alone or in combination with each other or with one or more other atheroprotective drugs, provided that they do not negatively interfere with each others action. A medical professional will know which drugs and combinations to choos .

The 7α-methyl-17α-ethynyl-estrane derivatives according to the invention may be administered enterally or parenterally and for humans in a daily dosage of 0.05 - 10 mg, preferably 0.1-2.5 mg.

A daily dose can be administered in one or more dosag units through for example th oral, the rectal, the sublingual rout , the nasal route or through

th skin (for xampl , transd rmal patches, or in th form f a cr am). Preferably a single dosage unit a day is administ r d by the oral rout . Alternatively, a controlled release preparation, releasing the daily required total dose as defined above, can be used. Controlled release preparations can be taken by the oral route, or are preferably applied in the form of a subcutaneous implant.

The pharmaceutical preparations for use according to the invention can be prepared in accordance with standard techniques such as for example are described in the standard reference, Gennaro et al. (Ed.), Remmington's Pharmaceutical Sciences, (18th ed. Mack Publishing Company, 1990, e.g. Part 8: Pharmaceutical Preparations And Their Manufacture). For the purpose of making the pharmaceutical preparations according to the invention, the 7α-methyl-17α-ethynyl-estrane derivatives according to formula I, or pharmaceutically acceptable salts thereof, are mixed with or dissolved in a pharmaceutical acceptable carrier. Examples of such preparations are tablets, pills, suppositories, (micro-)capsules, powders, emulsions, creams, ointments, suspensions, solutions, implants, or sprays.

Examples of pharmaceutically acceptable carriers are: starch (for example potato or corn starch), sugars (for example lactose), lubricants (for example magnesium stearate), binders (for example amylopectine or polyvinyl pyrrolidone), water, alcohol, glycerol and its derivatives, vegetable-, animal- and mineral oils and fats, fatty alcohols, silicones, polyalkylene glycols, cellulose derivatives, silica, dispersants, emulsifiers, surfactants, anti-oxidants, colorants and preservatives. In fact, any conventional pharmaceutical carrier that does not interfere with performance of the active ingredient can be used in the preparations according to the present invention.

Pharmaceutical preparations of the preferred 7α-methyl-17α-ethynyl-estrane derivative of the invention, i.e. 7α-methyl-17α-ethynyl-17β-hydroxy-estra-5(10)-en-3-one (Org OD-14; tibolone), are preferably prepared using the crystalline pure monoclinic ($P2_1$) form of Org OD-14, because of its improved stability, bioavailability and shelf-life. The synthesis and use in a pharmaceutical preparation of this monoclinic derivative of Org OD-14 is disclosed in European Patent No. 0,389,035B1.

The invention is illustrated by the following examples:

General

The atheroprotective properties of 7α-methyl-17α-ethynyl-estrane derivatives according to formula I are revealed in a cholesterol fed rabbit model wherein the effects of the compounds on the atherogenesis in female ovariectomized rabbits are established. The model is considered relevant to the human atherosclerotic process, because the cellular events occurring during the development of the atherosclerotic lesions during the atherogenic diet are similar to those observed in different stages of atherosclerotic processes in coronary arteries. Rabbits, however, do not have Lp(a) in their plasma.

Example 1

Tablets were prepared from a basic granulate containing lactose (100 mg per tablet) and dried potato starch (10 mg per tablet). The base granules were prepared by mixing the lactose with a portion of the starch. The remainder of the starch was mixed to a slurry with water and added to the mixture. The whole was granulated and dried. These base granules were mixed with ascorbyl palmitate (0.2 mg per tablet) and with either one of OD14 (2 mg or 6 mg per tablet) or 17β-estradiol (4 mg per tablet), sieved, finely mixed with magnesium stearate (0.5 mg per tablet) and then tabletted.

Example 2.

An experiment was performed with 7 groups of sexually matur , virgin female New Zealand White rabbits (Harlan, Zeist, The Netherlands), age 7-9 months and weighing approximately 3 kg (number of rats per group: 13-14). During the acclimatization period the rabbits were fed a diet of standard commercial rabbit chow LKK20 (Hope Farms, Woerden, The Netherlands). Three we ks prior to the start of the experiment the animals were anaesthetized and underwent bilateral ovariectomy (OVX) or were sham operated. After three weeks, at the start of the experiment, the rabbits were randomized over the treatment groups. In all animals de-endothelialisation of a segment of the left carotid artery was applied by using the air-drying technique (Fishman, J.A. et al., "Endothelial regeneration in the rat carotid artery and the significance of endothelial denudation in the pathogenesis of myointimal thickening", Lab. Invest. 1975, 32, 339-351; and Lafont, A. et al., "Restenosis and experimental angioplasty: Intimal, medial and adventitial changes associated with constructive remodeling", Circulation Research, 1995, 76, 996-1002)

The animals were randomly allocated into 7 experimental groups using a randomized block design. The groups were fed an atherogenic diet (commercial rabbit chow (LKK20) enriched with 0.4 grams cholesterol, 3.75 grams coconut oil and 3.75 grams peanut oil per 100 grams). One group was fed the standard rabbit chow (LKK20). Food intake was restricted to 80 grams daily.

The treatments (Table I) were daily administered orally as a tablet, prepar d as in Example 1. Groups 1, 2 and 3 were on a daily placebo treatment. Groups 4-5 were treated with 7α-methyl-17α-ethynyl-17β-hydroxy-estra-5(10)-en-3-one (OD-14; tibolone; 6 or 2 mg daily); group 6 with 17β-estradiol (4 mg daily). Group 7 was on treatment with 17β-estradiol decanoate (150 µg in 1 ml arachis oil injected subcutaneously once a week).

Table I: Design of the experiment

Group	n	Treatment*	Dose	Diet	OVX
1	14	Placebo		cholesterol	Yes
2	13	Placebo		cholesterol	No
3	13	Placebo		normal	Yes
4	14	Org OD14	6 mg	cholesterol	Yes
5	13	Org OD14	2 mg	cholesterol	Yes
6 E2	14	Estradiol	4 mg	cholesterol	Yes
7 E2D	14	Estradiol decanoate	150 µg	cholesterol	Yes

*The doses were administered orally except for group 7 in which the dose was injected subcutaneously once a week.

During the experiment blood samples were drawn out of the central ear artery after sedation with Hypnorm (0.1 ml i.m.) (Janssen Pharmaceutics, Beerse, Belgium) before the daily treatment, at week 4,8,12,16 and 20, to monitor the plasma cholesterol levels and to measure, at week 17, plasma estradiol levels and plasma tibolone levels.

Twenty weeks after the start of the experiment animals were anaesthetized by an i.m. injection of Hypnorm (0.5 ml/kg). After blood sampling the rabbits were killed by exsanguination and the aortic arch, uterus and carotid artery removed for further analyses.

Example 3

In addition to example 2 an additional experiment was performed with two lower doses of Org OD14 and a higher dose of estradiol decanoate. Thes groups together with a placebo group and a control group resulted in an experiment with 5 groups (see table II).

The procedures wer identical to thos in example 2.

Thr weeks prior to the start of th experiment th animals were ovariectomized. At the start of th xp riment the carotid artery was d - endothelialised using the air-drying technique. The normal rabbit chow was replaced by the atherogenic diet except for the control group which received during the experiment the normal rabbit chow.

Table II: Design of the experiment

Group	n	Treatment*	Dose	Diet	OVX
1	22	Placebo		cholesterol	Yes
2	13	Placebo		normal	Yes
3	13	Org OD14	0.6 mg	cholesterol	Yes
4	11	Org OD14	0.15 mg	cholesterol	Yes
5 E2D	14	Estradiol decanoate	300 µg	cholesterol	Yes

*The doses were administered orally except for group 5 in which the dose was injected subcutaneously once a week.

The treatments (table II) of group 1, 2, 3 and 4 were daily administered orally as a tablet, prepared as in example 1. The doses of Org OD14 per tablet, however, were 0.6 mg and 0.15 mg respectively. Group 5 was on treatment with 17β-estradiol decanoate (300 µg in 1 ml arachis oil injected subcutaneously once a week).

Twenty weeks after the start of the experiment was ended in the same manner as described in example 2.

EVALUATION OF ATHEROSCLEROSIS

A: Fatty streaks

The aortic arch was dissected free, opened longitudinally and fixed in 2% paraformaldehyde. The tissue was then stained for lipids using 0.3% (w/v) Sudan Red. Colour photographs were taken of all segments. The percentag coverage of the aortic arch (Table II) with fatty streaks was assessed using image analysis (Context Vision Systems AB, Linköping, Sweden).

B: Vessel wall cholesterol measurement

After fatty streak measurement the aortic arch was minced in a dismembrator (Mikro-Dismembrator, B. Braun, Melsungen, Germany), followed by a lipid extraction according to the method of Bligh and Dyer (Can. J. Biochem. Biophys. 1959, 37, 911-917). The total cholesterol content in the chloroform/methanol extraction solution was, after evaporation under nitrog n and dissolution in methanol, determined using enzymatic CHOD-PAP method (cat. no. 1442341, Boehringer Mannheim, Germany) and evaluated in a spectrophotometer (wavelength 500 nm). The amount of protein in the tissu was determined by the method of Lowry.

C: Intimal thickening after de-endothelialisation

The left (airdried) carotid artery was dissected and fixed in 2% paraform-aldehyde containing 6.8% glucose. The right carotid artery was used as comparison. After fixation the tissue was divided in blocks with a length of 2 mm and embedded in paraffin (Paraplast plus®, Sherwood Medical Co, St. Louis, USA) using an automated tissue processor (Hypercenter XP, Shandon).

To measur intimal thick ning (using image analysis) a specific staining method and a belonging imag analysis application have b en develop d.

Measurements were performed on 2 µm transverse sections which were treated with lastase (Serva Feinbiochemica Gmbh, Heidelberg, Germany) prior to elastin staining with Lawson solution (Boom, Meppel, the Netherlands) and light green (Sigma). Subsequently sections were airdried and mounted in Pertex (Leica Gmbh, Nussloch, Germany).

For morphological study both methylene blue/Azur II and hemotoxylin/eosin stained (2 µm) transverse sections were used. Smooth muscle cells and macrophages were detected with respectively α-actin antibodies (Sigma) and anti-macrophage antibodies (RAM11, DAKO, Glostrup, Denmark). For detection of bound antibodies goat anti-mouse ultra small gold conjugated secondary antibodies (Aurion, Wageningen, The Netherlands) and the immunogold-silver enhancement technique (SilvEnhance-LM Kit, Zymed) were used.

Images of the sections were obtained using a black and white video camera. (MX-5 , Adimec Image Systems BV, Eindhoven) mounted upon a light microscope (Axioplan, Zeiss, Jena, Germany). The video image was digitized and the intimal thickening was measured using a semi-automated image analysis application (Context Vision Systems AB, Linköping, Sweden).

Statistics: Data were expressed as mean ± S.E.M. unless otherwise specified. For testing statistical significance the Analysis of Variance (ANOVA) was used. The data were logarithmically transformed to normalize variations. A value of $P<0.05$ was considered to be significant.

RESULTS (xample 2, Tabl III)

After 20 weeks of diet and of daily treatment, necropsy was performed to determine the accumulation of cholesterol and fatty streaks in the aortic arch and the advanced lesions in the carotid artery. Moreover the weight of the uterus was determined. The results are presented in the Table III.

No significant difference in any of the variables measured were found between the ovariectomized animals (group 1) and the non-ovariectomized animals (group 2). This confirms that in the non-ovariectomized female rabbits the endogenous basal plasma estradiol levels are low.

Oral administration of 17β-estradiol (4 mg per day; group 6; E2) resulted in peak plasma estradiol levels of 238 pg/ml at 1h after administration, which was reduced to 18 pg/ml after 24 hours. Subcutaneous administered estradiol decanoate (150 µg per week; group 7; E2D) resulted in rather stable plasma estrogen levels over the day (about 60 to 70 pg/ml). The plasma levels indicate that both modes of administration of estradiol lead to effective plasma levels.

Using the human doses of estradiol and Org OD14 used for HRT treatment and corrected for the caloric intake it was expected that in the rabbit an oral dose of 4 mg estradiol is about equipotent with 6 mg Org OD14. Measurement of the plasma levels in the rabbit showed that Org OD14 at a dose of 6 mg per day resulted in plasma concentrations of Org OD14 comparable to those obtained in women with the clinical dose of 2.5 mg.

Org OD14 (2 and 6 mg per day) increased uterus (estrogenic activity) weight to a similar extent as both estradiol treatments (E2 or E2D), confirming the equipotency of the doses us d.

Despite this equipotent activity of the estrogen and Org OD14 treatments on the uterus the effect on cholesterol accumulation in the aorta and lesion formation in the aorta were different: 17β-estradiol (E2) treatment did not lead to a reduction in cholesterol accumulation, 17β-estradiol decanoate(ED2) treatment reduced cholesterol accumulation in the aortic arch with 46%, while Org OD 14 completely prevented cholesterol accumulation in the aortic arch at both concentrations studied.

17β-estradiol (E2) and 17β-estradiol decanoate (E2D) did not affect plasma cholesterol levels while Org OD14 strongly reduced the plasma cholesterol levels.

Fatty streak formation in the aortic arch was only slightly reduced by estrogen treatment while Org OD14 (nearly) completely prevented the fatty streak formation.

17β-estradiol (E2) had no effect on advanced lesion formation, following mechanical de-endothelisation, while 17β-estradiol decanoate (E2D) reduced advanced lesion formation. Histology showed that in placebo animals on a cholesterol diet the lesions consisted of smooth muscle cells and foam cells while in animals on a normal diet only smooth muscle cell were observed in the lesions. Histology of the E2D treated animals showed that the lesions still consisted of smooth muscle cells and foam cells. Org OD14 strongly inhibited the formation of advanced lesions. Lesion formation was even less than in the animals on a normal diet (control group). Histology showed that the lesions consisted only of smooth muscle cells.

RESULTS (example 3, Table IV)

In example 2 we found that 2 and 6 mg Org OD14 in the rabbit strongly inhibited the development of atherosclerotic lesion formation. These effects were unexpectedly much more pronounced than of estradiol or estradiol decanoate while there was an equipotent estrogenic activity on the uterus.

In example 3 we tested Org OD14 at two lower doses (0.6 and 0.15 mg orally once daily) and estradiol decanoate at a higher dose (300 µg s.c. once weekly). The aim was to obtain a dose of Org OD14 which had equipotent anti-atherosclerotic effects as estradiol decanoate.

The results show that Org OD14 at 0.6 mg still nearly completely prevented atherosclerotic lesion formation. A two times higher dose of estradiol decanoate (compared to table III) still only partially inhibited atherosclerotic lesion formation.

A dose between 0.60 mg-0.15 mg Org OD14 will show about equipotent anti-atherosclerotic effects as the 300 µg dose of estradiol decanoate. The effect on the uterus, however, was completely different. While the uterus weight increased from 0.054 to 0.132 grams (144 % increase) for Org OD14 it increased to 0.505 grams (835% increase) per 100 grams body weight for estradiol decanoate.

CONCLUSION

The results of example 2 demonstrate that while 17β-estradiol, 17β-estradiol decanoate and Org OD14, in clinically equivalent doses, are approximately equally potent on the uterus growth in rabbits, the beneficial effects of OD14 on plasma cholesterol, cholesterol accumulation in the aortic arch and advanced lesion formation in the carotid artery, are much more pronounced than those of 17β-estradiol.

Furthermore, as demonstrated in example 3, at a dose between ten to forty times lower (0.60-0.15 mg) Org OD14 had about equipotent anti-atherosclerotic effects as a two times higher dose of estradiol decanoate (300 µg). The estrogenic effect (increase in uterus weight) for estradiol decanoate, however, was much more pronounced than for Org OD14.

These results indicate that Org OD14 has intrinsically the potential to be clinically effective for the prevention and treatment of atherosclerosis.

Table III

The effect of different treatments on the atherogenic diet induced morphologic changes in the aortic arch and carotid artery as well as the influence on body weight, plasma cholesterol and uterus weight as measured at the time point of necropsy (20 weeks after the start of the experiment).

	Controle Group 3	Placebo Group 1	Org OD14 Group 4	Org OD14 Group 5	Estradiol Group 6	Estradi l decan at Group 7
Number of Rabbits	13	9	13	12	13	11
Body weight in grams	2831 ± 50	2683 ± 95	2758 ± 41	2815 ± 50	2831 ± 58	2973 ± 52
Uterus weight per 100 grams body weight	0.08 ±0.01	0.09 ±0.01	0.24 ±0.06 *	0.29 ±0.03 *	0.31 ±0.02 *	0.37 ±0.04 *
Mean plasma cholesterol exp sure in mmol/L/day	1.0 ± 0.1 *	26.9 ± 3.3	11.2 ± 1.5 *	9.0 ± 1.3 *	27.7 ± 2.8	30.0 ± 3.7
Cholesterol level in aortic arch in nmol/mg protein	45 ± 5 *	590 ± 126	52 ± 9 *	61 ± 11 *	521 ± 99	318 ± 58 *
Fatty streak in aortic arch in % of arcus covered	0.3 ± 0.2 *	34.5 ± 6.8	1.3 ± 0.8 *	1.4 ± 0.7 *	25.5 ± 5.2	28.1 ± 4.1
Advanced lesions in carotid artery: intimal surface in mm^2	0.22 ± 0.03 *	0.44 ± 0.10	0.13 ± 0.03 *	0.14 ± 0.02 *	0.30 ± 0.06	0.23 ± 0.06 *

* p< 0.05 compared with placebo (group 1)

Table IV

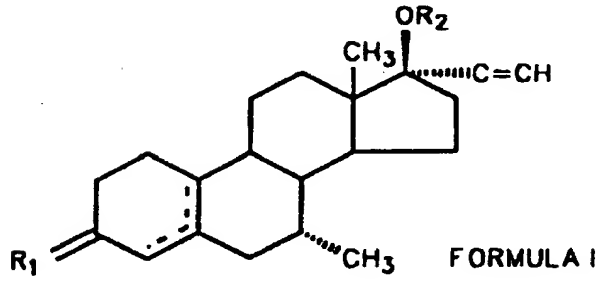
The effect of Org OD14 (0.6 and 0.15 mg orally per rabbit once daily) compared to a two times higher dose of estradiol d canoate (300 µg subcutaneously per rabbit once weekly) compared to the dose in table III on the atherogenic diet induced morphologic changes in the aortic arch and carotid artery as well as the influence on body weight, plasma cholesterol and uterus weight as measured at the time point of necropsy (20 weeks after the start of the experiment).

	Controle	Placebo	Org OD14 0.6 mg	Org OD14 0.15 mg	Estradiol decanoate
Number of Rabbits	13	22	13	11	14
B dy weight in grams	2869 ± 38	2882 ± 73	3008 ± 58	2850 ± 42	3093 ± 60
Uterus weight per 100 grams body weight	0.051 ±0.009	0.054 ±0.007	0.224 ±0.016 *	0.132 ±0.013 *	0.505 ±0.075 *
M an plasma cholesterol exposure in mmol/L/day	1.3 ± 0.4 *	38.9 ± 2.5	12.8 ± 1.2 *	20.7 ± 1.5 *	23.0 ± 2.0 *
Ch lesterol level in aortic arch in nmol/mg protein	51 ± 9 *	636 ± 53	97 ± 23 *	297 ± 67 *	225 ± 69 *
Fatty streak in aortic arch in % of arcus covered	1.0 ± 0.7 *	41.9 ± 2.8	5.9 ± 2.4 *	23.5 ± 5.3 *	14.2 ± 3.2 *
Advanced lesions in carotid artery: intimal surface in mm^2	0.13 ± 0.02 *	0.31 ± 0.07	0.14 ± 0.01 *	0.24 ± 0.05	0.18 ± 0.02 *

* $p < 0.05$ compared with placebo (group 1)

Claims:

1. Use of a 7α-methyl-17α-ethynyl-estrane derivative having the general formula I



FORMULA I

wherein

R_1 = H(OR_3) or O;

R_2 = H or (C_{1-18})Acyl;

R_3 = H or (C_{1-18})Acyl;

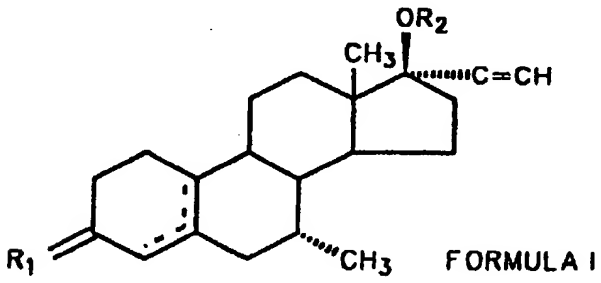
and the dotted line represents a double bond in the 4,5- or the 5,10-position, for the manufacture of a medicament for the prophylaxis or the treatment of atherosclerosis.

2. Use according to claim 1, wherein R_1 = H,OH or O.

3. Use according to claim 1 or 2, wherein R_2 = H and the dotted line represents a double bond in the 5,10-position.

4. Use according to claim 1, wherein the a 7α-methyl-17α-ethynyl-estrane derivative is 7α-methyl-17α-ethynyl-17β-hydroxy-estra-5(10)-en-3-one.

5. A method of inhibiting th process of atherosclerosis comprising administering to a mammal an ath roprot ctive amount of a 7α-methyl-17α-ethynyl-estrane derivative having the general formula I



FORMULA I

wherein

R_1 = H(OR_3) or O;

R_2 = H or (C_{1-18})Acyl;

R_3 = H or (C_{1-18})Acyl;

and the dotted line represents a double bond in the 4,5- or the 5,10-position.

6. The method according to claim 5 wherein the mammal is human.